

Attorney Docket: 950376/HG

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of : Tohru TAKAHASHI et al  
Serial No. : 08/500,635  
Filed : July 11, 1995  
For : EXPRESSION SYSTEMS UTILIZING  
AUTOLYZING FUSION PROTEINS  
AND A NOVEL REDUCING POLYPEPTIDE  
Art Unit : 1814  
Examiner : Dr. L. Hobbs

DECLARATION UNDER 37 CFR 1.132

I, Tohru Takahashi, of 4-21-23, Takanodai, Nerima-ku, Tokyo, Japan, a citizen of Japan, sincerely and solemnly declare as follows:

1. I am a graduate of the graduate school of Hokkaido University where I majored in plant virology, culminating in Doctor's Degree in Agriculture. I am now an Assistant Chief Researcher of Biomedical Research Laboratories of Sankyo Company, Limited, Tokyo, Japan having entered into the employment of the said company in April, 1990 after graduating from the said graduate school in March, 1990. I am a member of the Molecular Biology Society Japan.

I have published a number of the scientific papers, including:

(1) Journal of General Virology, 70, 1297-1300 (1989):

"Nucleotide Sequence of Rice Dwarf Virus Genome Segment 9"

(2) Intervirology, 32, 234-245 (1991):

"Relatedness of the Nucleotide sequence of the 3'-Terminal Region of Clover Yellow Vein Potyvirus RNA to Bean Yellow Mosaic Potyvirus RNA"

(3) Virus Genes, 14, 235-243 (1997):

"A cDNA Clone to Clover Yellow Vein Potyvirus Genome is Highly Infectious"

2. I understand that the above application has been rejected under 35 USC §103 as being unpatentable over Chang et al, Boye et al, Dogherty et al, Sommergruber et al and Hopp et al.

3. In order to overcome the obviousness rejection under 35 USC §103 by showing that these prior art references would not be combined by a person of ordinary skilled in the art, I carried out the following experiments.

### **Comparison of Expression Systems Using Potyvirus Nuclear Inclusion a Proteins.**

#### **Materials, Methods and Results**

##### **cDNA synthesis and Molecular cloning**

Isolate No.30 of clover yellow vein virus (CYVV) was originally obtained from Phaseolus vulgaris and propagated in

Vicia faba in a greenhouse <sup>1)</sup>. A necrotic strain of of potato virus Y (PVY) was also originally isolated from potato plants and propagated in Nicotiana sylvestris <sup>2)</sup>. The viruses were purified from the infected plants using the method previously described <sup>1)</sup>, and the viral RNA was extracted by an alkaline sucrose density gradient method <sup>3)</sup>. Complementary DNA synthesis and molecular cloning were done according to the method of Gubler and Hoffman <sup>4)</sup>.

### Nucleotide sequence determination

The resultant clones, pNS51 from CYVV and pPVTdT88 from PVY-N, were subcloned into M13mp18 and M13mp19, and a series of sequential deletions of the inserts were made for sequencing using Exonuclease-III. Sequences were determined by the dideoxy-chain termination method.

### Isolation of the NIa structural gene

The NIa gene was isolated from pNS51 by use of the polymerase chain reaction method (PCR), and the human interleukin-11 (hIL-11) gene was isolated from pcD20-2 carrying hIL-11 cDNA <sup>5)</sup>. These genes were joined with a specific linker that coded for a proteolytic processing amino acid sequence (Gln-Ala), and were inserted into E. coli expression vector pKK388-1. The resulting vector, pKSun9, which carried the NIa-Gln-Ala-hIL-11 fused gene, was expressed by the trc promoter (Fig. 1). For comparison with other expression systems, we also prepared a mature hIL-11 type vector with an additional initiation codon

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(pMIL), and other constructs of the NIa-fusion vector (Fig. 1). Another type of NIa was isolated from a necrotic strain of potato virus Y (PVY)<sup>21</sup>, which is a well-known type member of the genus Potyvirus. In the case of glutathione S-transferase (GST)- or other protein fusion systems, we found that the N-terminus proline cluster of hIL-11 inhibited protease cleavage (data not shown). Consequently, we made two types of expression vectors from PVY-NIa. The p $\Delta$ PVIL expression vector has the PVY-NIa original cleavage site, but some extra sequences inserted upstream of the proline cluster-deficient hIL-11. On the other hand, pPVIL was constructed in the same way as pKSun9.

#### Expression of hIL-11 by the auto-cleavage system of NIa

The expression vector plasmid pKSun9 and others were used to transform E. coli JM109 (hereinafter, the E. coli clones containing these plasmids are abbreviated as KSUN9, MIL,  $\Delta$ PVIL and PVIL). The methionine-fused hIL-11 or NIa/hIL-11 fusion proteins were expressed by induction with 1mM isopropylthio-b-D-galactoside (IPTG). The expressed recombinant proteins were analyzed by western blotting. Cell extracts were subjected to blotting with an anti-hIL-11 polyclonal antibody. Twenty-three-KDa and 60-KDa protein signal bands, specific to the cell extract of KSUN9, were detected (Fig. 2). The 23-KDa band migrated at the same rate as mature hIL-11. In the case of  $\Delta$ PVIL, we also detected two bands, one at 25-KDa and one at 60-KDa. In contrast, only the 60-KDa band was detected in the cell extract of PVIL, and no band was detected in the cell extract of MIL.

## Conclusion

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In these experiments, we investigated the use of two typical potyviruses: potato virus Y<sup>2)</sup> and clover yellow vein virus<sup>1,6)</sup>. To investigate whether these proteases could process the NIa and hIL-11 fused protein, we made PVY-NIa/hIL-11 and CYVV-NIa/hIL-11 fusion protein expression vectors. In these experiments, we succeeded in the direct expression of hIL-11 when CYVV-NIa (E. coli JM109/KSUN9) was used. On the other hand, when PVY-NIa (E. coli JM109/PVIL) was used, no hIL-11 was processed from the fusion protein. Although we also examined the use of the GST fusion system for rhIL-11, complete cleavage of the fusion protein was difficult using enterokinase. In comparison to PVY-NIa, CYVV-NIa has higher specificity and broader utility than other potyvirus proteases, and therefore it can be used in other fusion protein expression systems. In fact, we succeeded to produce other clinically important proteins like the KM-102 derived reductase like-factor, interferon and some allergenic antigens (data not shown). These proteins are also difficult to produce by commonly used expression systems.

## References

- 1) Uyeda, I., Kojima, M. and Murayama, D. (1975) Ann. Phytopath. Sic. Japan 41, 192-203.
- 2) Hataya, T., Sano, T., Ohoshina, K. and Shikata, E. (1990) Virus gene 4, 339-350.
- 3) Dougherty, W. G. and Hiebert, E. (1980) Virology 104, 183-

194.

- 4) Gubler, U. and Hoffman, B. J. (1983) *Gene* 25, 263-269.
- 5) Kawashima, I., Ohsumi, J., Mita-Honjo, K., Shimoda-Takano, K., Ishikawa, I., Sakakibara, S., Miyadai, K. and Takiguchi, Y. (1991) *FEBS Lett.* 283, 199-202.
- 6) Uyeda, I., Takahashi, T. and Shikata, E. (1991) *Intervirology* 32, 234-245.

### Figure to Legend

Fig. 1. Schematic of hIL-11 expression system under direct or N1a-fusion systems.

Each gene is inserted into the *E. coli* expression vector pKK388-1, and promoted by the *tac* promoter. Plasmid pMIL is simply one with ATG in front of the N-terminal proline of the mature hIL-11. Plasmid pΔPVIL contains PVY-N1a and hIL-11 genes joined in the same reading frame, but in this plasmid, the hIL-11 gene is incomplete (lack from +1 to +7: N'-PGPPPGP-C'). Plasmid pΔPVIL has an original specific cleavage site and 55 amino acids of the N terminus of N1b. These genes are fused in the same reading frame. Plasmid pPVIL has an original specific cleavage site and joined in the same reading frame with complete hIL-11 gene.

Fig. 2. Western analysis of the expressed recombinant hIL-11.

Lane 1: whole cell extract of *E. coli* JM109 containing no plasmid; Lane 2: MIL; Lane 3: KSUN9; Lane 4: PVIL; Lane 5:

ΔPVIL. These samples were electrophoresed on a SDS-PAGE and then immunoblotted with anti hIL-11 rabbit polyclonal antibody. The positive control (lane 7: 50ng, and 8: 100ng) was purified from recombinant S. picha, which expressed recombinant hIL-11 (lacking N-terminal proline).

4. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 15. Dec. 1997

By:   
Name: Tohru Takahashi

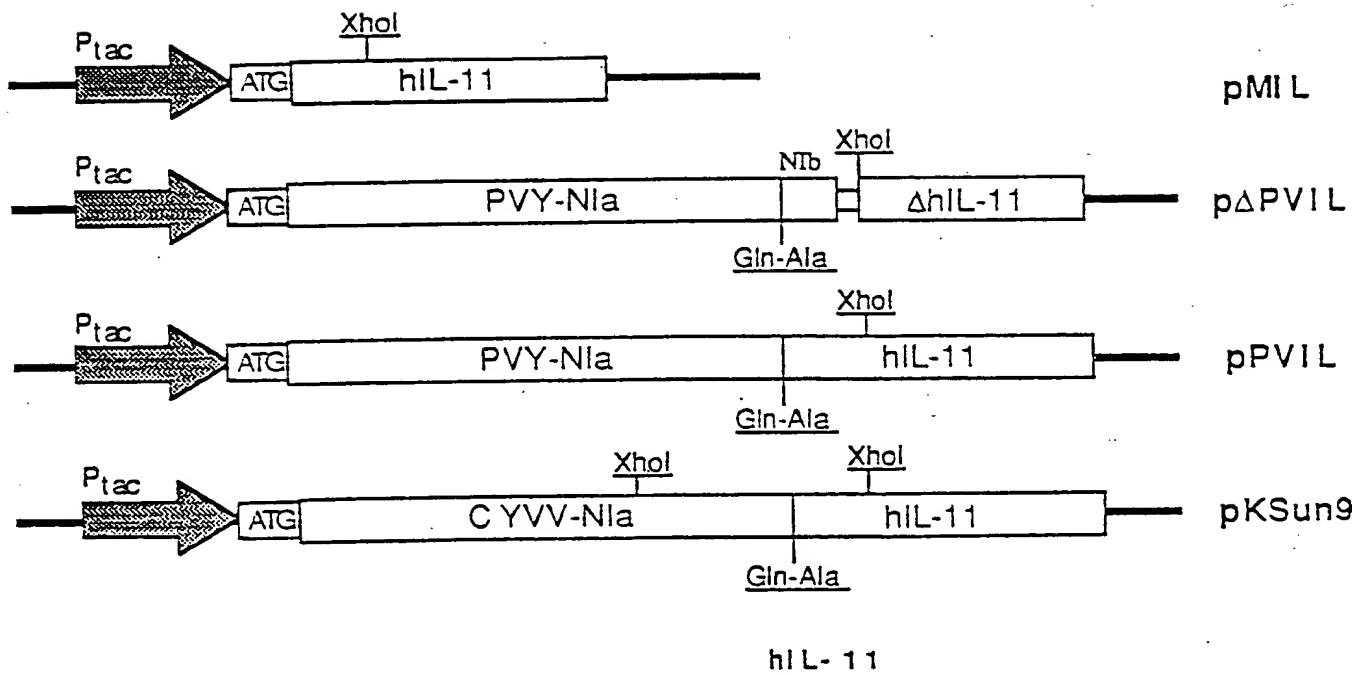


Fig. 1

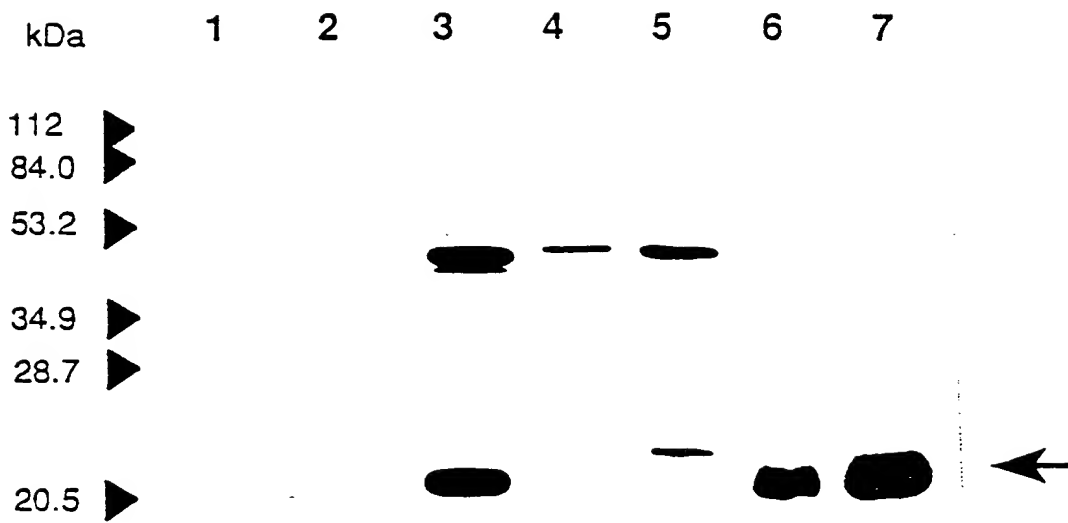


Fig. 2